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Short communication

Preparative isolation and purification of calycosin from *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao by high-speed counter-current chromatography

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Abstract

Calycosin was purified from an ethyl acetate extract of the root of *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao by high-speed counter-current chromatography. The separation was performed in two steps with a two-phase solvent system composed of *n*-hexane–chloroform–methanol–water (1:3:3:2, v/v). From 200 mg of the crude extract, 14.8 mg of calycosin was obtained at over 99% purity as determined by HPLC analysis, and its chemical structure was confirmed by MS, ^{1}H and ^{13}C nuclear magnetic resonance. Published by Elsevier Science B.V.

Keywords: *Astragalus mongholicus*; Preparative chromatography; Counter-current chromatography; Calycosin

1. Introduction

The roots of *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao, and certain species of *Astragalus* (Leguminosae) have long been used as an anti-perspirant, adiuretic or atonic in Chinese traditional medicine under the name of Huang-qi in China (Ougi in Japan) [1–4]. The pharmacologic studies and clinical practice have demonstrated its immunostimulant, cardiotonic and antiaging activities. Its isoflavones, including calycosin showed antimicrobial and superoxide anion scavenging activities [5–7]. Since the flavonoids are known as one of the major beneficial components [2–4,8–10] with suitable chromophores for UV detection, they have been chosen as ‘‘marker compounds’’ for the chemical evaluation or standardization of Huang-qi and its products [4,11,12].

Calycosin is the main isoflavone component of *A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao. It is proved to be active against *Giardia intestinalis*, a potent protozoan agent for enteric disease [13]. It has also been shown to possess in vitro activity against the chloroquine-sensitive strain poW and the chloroquine-resistant clone Dd2 of *Plasmodium falciparum* [14].

The preparative separation and purification of calycosin from plant materials by conventional meth-

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Fig. 1. Chemical structure of calycosin.

ods is tedious and usually requires multiple chromatography steps such as column chromatography and high-performance liquid chromatography (HPLC). High-speed counter-current chromatography (HSCCC) [15], being a support-free liquid–liquid partition chromatography, can eliminate irreversible adsorption of sample onto the solid support, and therefore is considered as a suitable alternative for the separation of phenolic compounds such as flavonoids and hydroxyanthraquinones [16–18].

The present paper introduces a method for the purification of calycosin (see Fig. 1) from a crude extract of *A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao by HSCCC.

2. Experimental

2.1. Apparatus

The analytical HSCCC instrument employed in the present study is a Model GS 20 analytical high-speed counter-current chromatograph designed and constructed in Beijing Institute of New Technology Application (Beijing, China). The apparatus holds a pair of column holders symmetrically on the rotary frame at a distance of 5 cm from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 50 m×0.85 mm I.D. PTFE (polytetrafluoroethylene) tube directly onto the holder hub forming multiple coiled layers with a total capacity of 40 ml. The β value varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). Although the revolution speed of the apparatus could be regulated with a speed controller in the range between 0 to 2000 rpm, an optimum speed of 1800 rpm was used in the present studies.

Preparative HSCCC was performed using a Model GS10A2 multilayer coil of 110 m×1.6 mm I.D. with a total capacity of 230 ml. The β values of this preparative column range from 0.5 to 0.8.

The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application). Continuous monitoring of the effluent was achieved with a Model 8823A-UV monitor (Beijing Institute of New Technology Application) at 254 nm. A manual sample injection valve with a 1.0-ml loop (for the analytical HSCCC) or a 20-ml loop (for the preparative HSCCC) (Tianjin High New Science Technology, Tianjin, China) was used to introduce the sample into the column, respectively. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

The HPLC equipment used was a Shimadzu LC-10AVP system including two LC-10ATVP solvent delivery units, an SPD-M10AVP UV–Vis photodiode array detection (PAD) system, an SC-10AVP system controller, a CTO-10ASVP column oven, a DGU-12A degasser and a Class-VP-LC workstation (Shimadzu, Kyoto, Japan).

2.2. Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. Acetonitrile used for HPLC analysis was of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory, Tianjin, China.

2.3. Extraction of crude isoflavones

Raw roots of *A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao (47.5 kg) were extracted three times with 95% ethanol. Then, the extract was combined and evaporated to dryness under reduced pressure, which yielded 6.8 kg of dry powder. About

1 kg of the residue obtained from the combined extract was dissolved in 6 l of water. After filtration, the aqueous solution was extracted three times with 5 l water-saturated light petroleum (b.p. 60–90 °C), ethyl acetate and *n*-butanol, successively. By combining each group of extracts and evaporating to dryness under reduced pressure, we obtained 1 g of light petroleum extract, 25 g of ethyl acetate extract and 119 g of *n*-butanol extract. Portions of the above ethyl acetate extract of *A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao were subjected to HSCCC.

2.4. Preparation of two-phase solvent system and sample solutions

The two-phase solvent system used was composed of *n*-hexane–chloroform–methanol–water (1:3:3:2, v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use.

The sample solutions were prepared by dissolving the crude extract in the lower phase at suitable concentrations according to the analytical or the preparative purpose.

2.5. Separation procedure

The analytical HSCCC separation was performed with a Model GS 20 HSCCC instrument as follows: the multilayer-coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the column at a flow-rate of 1.0 ml/min, while the apparatus was run at a revolution speed of 1800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (10 mg dissolved in 1 ml of the upper aqueous phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram.

Preparative HSCCC was similarly performed with a Model GS 10A2 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the upper aqueous phase as stationary phase. Then the sample solution (200 mg dissolved in 20 ml of the lower organic phase) was injected through the sample port and the lower organic phase was pumped into the column at a flow-rate of 2.0 ml/min while the column was rotated at 800 rpm. The effluent from the outlet of the column was monitored with a UV detector at 254 nm. Peak fractions were manually collected according to the chromatogram.

2.6. HPLC analysis and identification of CCC peak fractions

The crude ethyl acetate extract of *A. mongholicus* and each HSCCC peak fraction were analyzed by HPLC. The analyses were performed with an Intersil ODS-3 column (150×4.6 mm I.D.) at a column temperature of 35 °C. The mobile phase composed of methanol–water (35:65, v/v) was eluted at a flow-rate of 1.0 ml/min, and the effluent monitored by (PAD).

Identification of the HSCCC peak fraction was carried out by mass spectrometry (MS), ^{1}H and ^{13}C nuclear magnetic resonance (NMR).

3. Results and discussion

The crude ethyl acetate extract of *A. mongholicus* was first analyzed by HPLC. The result indicated that it contained several compounds including calycosin (about 12%) and some unknown compounds as shown in Fig. 2A.

In order to achieve an efficient resolution of target compounds, the two-phase solvent system of *n*-hexane–chloroform–methanol–water was examined using analytical HSCCC by varying the mutual volume ratios. The result indicated that the volume ratio of 1:3:3:2 could separate calycosin well.

Fig. 3A shows the result obtained from 200 mg of the crude ethyl acetate extract of *A. mongholicus* by preparative HSCCC. After this separation the fractions containing calycosin (shaded peak) was collected. The HPLC analysis of this fraction indicated that it contained calycosin at over 75% purity (Fig. 2B). This partially purified fraction was dried, redissolved in the organic phase and purified by HSCCC with the same solvent system (Fig. 3B).

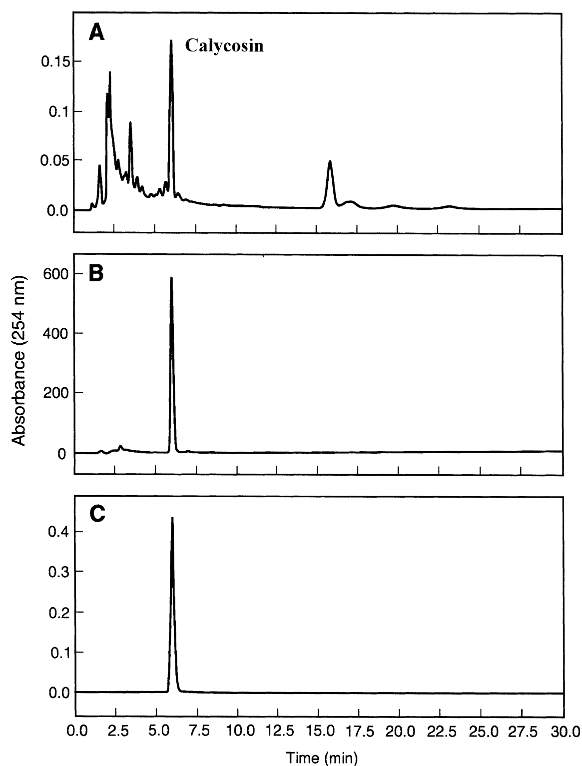

Fig. 2. HPLC analyses of the crude sample and HSCCC peak fractions. (A) Crude ethyl acetate extract from *A. mongholicus*; (B) HSCCC fraction corresponding to the calycosin peak (shaded) in Fig. 3A; (C) HSCCC fraction corresponding to the major peak in Fig. 3B. Experimental conditions are as follows: column: Intersil ODS-3 (150×4.6 mm I.D.); mobile phase: water–acetonitrile (65:35); flow-rate: 1 ml/min; detection at 254 nm.

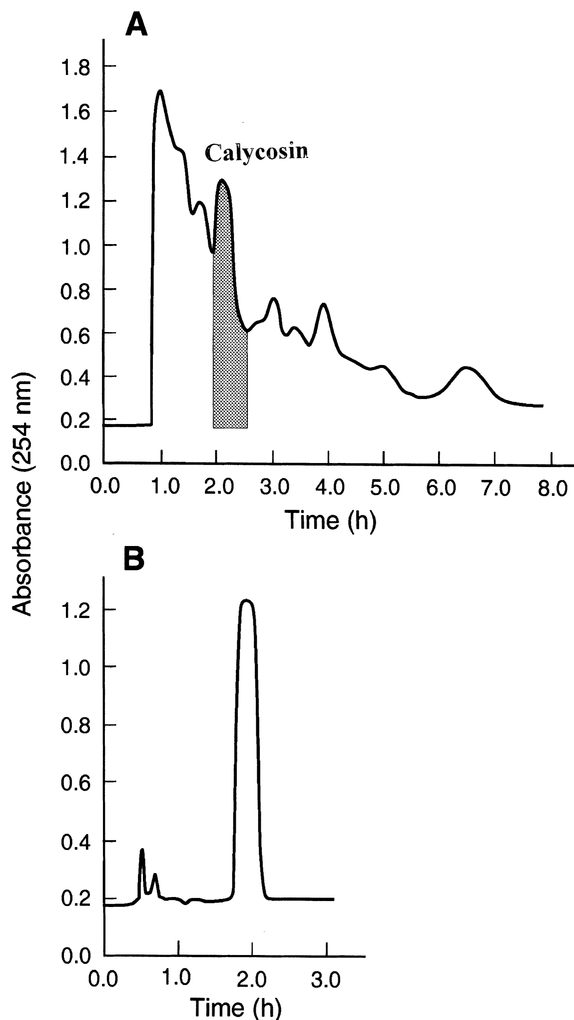

Fig. 3. Chromatograms of the crude ethyl acetate extract from *A. mongholicus* by preparative HSCCC. (A) First separation; (B) second separation of the fractions corresponding to the calycosin peak (shaded) of (A). Experimental conditions: apparatus: preparative HSCCC centrifuge with a multilayer coil separation column with 230 ml capacity; sample: (A) 200 mg of crude ethyl acetate extract of *A. mongholicus* dissolved in 20 ml of lower organic phase, (B) HSCCC fraction corresponding to the calycosin peak (shaded) of (A) dried and redissolved in the lower phase; solvent system: *n*-hexane–chloroform–methanol–water (1:3:3:2, v/v); mobile phase: lower organic phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; detection at 254 nm; retention of stationary phase: 66.7%.

This second separation yielded 14.8 mg of calycosin at over 99% purity as determined by HPLC analysis (Fig. 2C). The structural identification of the fraction was carried out by MS, ^{1}H- and ^{13}C-NMR.

The results of our studies clearly demonstrated that HSCCC is very useful for the preparative separation of calycosin from a crude extract of *A. mongholicus*.

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